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Introduction

The TP53 tumor suppressor gene is defective in the majority of sporadic breast cancers, and breast cancer is the most frequent tumor type in women with Li-Fraumeni syndrome who inherit germline mutations in TP53. This suggests that p53 is fundamental to the growth regulation and prevention of tumor formation in mammary epithelial cells. Our laboratory has backcrossed the p53-null allele in mice onto the BALB/c genetic background. We have recently described the occurrence of mammary tumors in 55% of female BALB/c-p53+/- mice with a latency of 8-14 months(1). This is in contrast to C57BL/6- and 129/Sv-p53+/- mice, which rarely develop mammary tumors (2), suggesting that the BALB/c-p53+/- mice serve as a unique model for Li-Fraumeni syndrome (LFS). The experiments proposed in this fellowship are designed to characterize the BALB/c-p53+/- mouse model of breast cancer with respect to the progression of the glands towards tumor formation, and with respect to genetic contributions towards tumor susceptibility which are particular to this strain of mouse.

Body

Specific Aim 1: Genetic alterations leading to mammary tumorigenesis in BALB/c-p53+/- mice. Sequential relationships between the loss of the wild-type allele of p53, appearance of genomic instability, and the development of mammary hyperplasias and tumors will be defined.

Task 1: To determine the earliest changes and monitor the progression of morphological and genetic changes occurring in the mammary glands of BALB/c-p53+/- mice.

Wild-type and p53+/- female mice were bred (task 1a) and aged for the collection of mammary gland tissues from mice at the ages of 20, 28, 36, 44, and 52 weeks and for the collection of mammary tumor tissues (task 1c). The PI has been trained in immunohistochemisty, southern blotting, primary culturing of mouse mammary epithelial cells and preparation of samples for karyotype analysis (task 1b).

DNA extracted from mammary tumors was analysed by southern blotting (Christy McLary, masters student under the supervision of the PI) for the presence of the wild-type and null p53 alleles. Of 25 mammary tumors examined, 24 (96%) showed significant loss of the wild-type allele, confirming that loss of the wild-type allele is a high frequency event in tumors arising in this model. Primary cultures were also prepared from mammary tumors for karyotypic analysis. Tumor cells were cultured for 3-4 days without passaging and nuclei preparations made, and the karyotypes of 5 tumors were examined (Dr Rizwan Naeem, collaborating cytogeneticist). The mammary tumors contained a hypodiploid population of cells lacking one copy of chromosome 11 as well as a near-tetraploid population of cells. These results demonstrate that aneuploidy and missegregation are occurring in the tumors, and suggest that the wild-type allele may be lost by missegregation and loss of one copy of chromosome 11. However, the possibility that loss of p53 occurs via interstitial deletion or recombination prior to missegregation cannot be excluded.

To determine the earliest morphological changes occurring in the mammary glands, a 4th gland from 3 mice of each genotype across the ages of 20 to 52 weeks was wholemounted and examined for gross morphological changes. No consistent difference was observed between wild-type and

p53+/- mammary glands. There was no evidence of the accumulation of hyperplastic / preneoplastic lesions in the aging p53+/- mice. Mammary gland tissues were also examined histologically (Prof D. J. Jerry, Mentor, and Dr Stephen Naber, collaborating pathologist). Consistent with the wholemounts, examination of mammary gland morphology on H&E stained sections did not reveal any consistent difference between wild-type and p53+/- mammary glands, and did not reveal any microscopic evidence of a reliable pre-neoplastic lesion arising during aging in p53+/- mice.

Primary cultures of mammary epithelial cells were also prepared from at least 6 mice of each genotype at each time point. These were cultured for 4 nights without passaging and metaphase nuclei preparations were made for the examination of karyotypic changes (breaks, gaps, dicentrics, excess acentric fragments) and aneuploidy. Initial analyses were done on the metaphase spreads from the 52 week old mice (Prof John Mailhes, collaborating cytogeneticist), examining 20-40 metaphases from at least 4 different mice of each genotype. Once again, there were no detectable differences between the wild-type and p53+/- mammary epithelial cells. In the absence of a detectable accumulation of chromosomal changes at 52 weeks of age, this analysis was not continued on the younger age groups.

Thus, while karyotypic changes were easily detected in the tumors, they did not occur at detectable levels in normal, aged p53+/- mammary epithelial cells. Loss of the wild-type allele of p53 is a consistent event in tumors, however, unless a preneoplastic population of cells can be identified in the normal p53+/- mammary glands, examination of the timing of the loss of p53 (component of task 1d) will be very difficult because of the high background of normal cells present. Thus, approaches to this problem will need to be reconsidered.

Experimental analysis of both normal and tumor tissues by immunohistochemistry for hormone receptor expression is continuing to further characterize this new tumor model.

Specific Aim 2: Mapping genetic modifiers of mammary tumor susceptibility in BALB/c-p53+/- mice. These experiments will lead to identification of loci that contain gene(s) that contribute to the higher susceptibility to mammary tumors in BALB/c- vs C57BL/6-p53+/- mice.

Task 2: To map the location of genetic modifiers of mammary tumor susceptibility in BALB/c-p53+/- mice.

A mapping panel of 226 female BALB-N2 p53+/- mice was bred (breeding scheme of [C57BL/6 x BALB/c] x BALB/c-p53-/-). In addition, 19 F1-p53+/- female mice were bred (C57BL/6 x BALB/c-p53-/-) (task 2a). These mice were monitored weekly for develpment of tumors, with special attention being paid to the appearance of mammary tumors.

As breeding had commenced prior to the activation of this grant, we are ahead of schedule on this task. The mice have been monitored for tumor development up to the age of 18 months and tissue collection has been completed (task 2b).

In the current absence of known preneoplastic markers in this model (see Aim 1), phenotypic analysis (task 2c) of the mice has been limited to the presence or absence of mammary tumors and the age of appearance of the first mammary tumor. Additional information has been collected, such

as the number of mammary tumors, and tissues for histological assessment of non-tumor bearing glands and wholemounts have been collected but not yet analyzed.

DNA has been isolated from tail tissue of all mice for genetic analysis (task 2d). Analysis of heterozygosity for BALB/c vs C57BL/6 alleles across the whole genome is underway in collaboration with Roche Biosciences, but no results are currently available from this analysis. We have completed an alternative analysis on two candidate genes where hypomorphic alleles have been identified in BALB/c mice and may be modifiers of tumor susceptibility --- DNA-PKcs (Prkdc) and the cyclin-dependent kinase inhibitor p16INK4A (Cdkn2a). Mice were genotyped at these two candidate loci ("B" = BALB/c; "+" = wild-type or C57BL/6) (Jennifer Brown, technician) and logistic regression tests performed on the Kaplan-Meyer mammary tumor-free survival curves (figure 1). Differences in mammary tumor occurrence were not statistically significant, however, a decrease in latency of 6-7 weeks was associated with Cdkn2aB/B genotype in mammary tumors occurring after 56 weeks of age, suggesting differences in the mechanism of tumorigenesis between early and late onset mammary tumors in the N2 population. These results identify Prkdc and Cdkn2a as modifiers of tumor latency in Trp53+/- mice but cannot account for the prevalence of mammary tumors in the BALB/c strain. The difference in mechanism between early and late onset mammary tumors will be taken into account when analyzing the genome scan data to assist in the identification of novel susceptibility loci.

Key Research Accomplishments

* Loss of the wild-type allele of p53 has been confirmed to occur in over 95% of mammary tumors arising in Trp53+/- mice of BALB/c genetic backgrounds, and thus is a consistent event in mammary tumorigenesis of this model.

* Karyotype analysis of mammary tumors has demonstrated aneuploidy and loss of one copy of chromosome 11, which could account for the loss of the wild-type allele of p53.

* Tumor formation is not preceded by gross morphological changes in the mammary gland, or by widespread detectable karyotypic changes in the mammary epithelial cells.

* Cdkn2a and Prkdc have been eliminated as major recessive contributors to the mammary tumor susceptibility of BALB/c-p53+/- mice.

* Cdkn2a has been identified as a modifier gene, altering the age of onset of mammary tumors in p53+/- mice by 6-7 weeks where mammary tumors arise with a latency of over 56 weeks.

* Genome scanning is underway in the N2 mapping population for mammary tumor susceptibility loci.

Reportable Outcomes

Publication:

Review article.
A. C. Blackburn and D. J. Jerry. Knock-out and transgenic mice of *Trp53*: what have we learned about p53 in breast cancer? Breast Cancer Res. (2002) 4:101-111. (Appendix 1)

Manuscripts in preparation:

A. C. Blackburn, S. P. Naber, J. S. Brown, and D. J. Jerry. *Prkdc* and *Cdkn2a* are genetic modifiers of lymphoma and *Cdkn2a* is a genetic modifier of mammary adenocarcinomas in Trp53+/- mice.

A. C. Blackburn, S. C. McLary, S. P. Naber, C. N. Otis, L. A. Donehower, T. Soferr, and D. J. Jerry.
Genetic background affects loss of heterozygosity and tumor spectrum in Trp53+/- mice.

Poster Presentations:

AACR Special Conference on Genetic Modifiers of Cancer Susceptibility, February 2001.
A. C. Blackburn, S. P. Naber, D. J. Jerry. Mapping genetic modifiers of mammary tumor susceptibility in BALB/c-p53+/- mice.

Mammary Gland Biology Gordon Conference, May 2001.
A. C. Blackburn, S. P. Naber, D. J. Jerry. Mapping genetic modifiers of mammary tumor susceptibility in BALB/c-p53+/- mice.

Molecular and Cell Biology of Cancer, 4th Peter Mac Symposium, Melbourne November 2001.
A. C. Blackburn, S. P. Naber, J. Brown, D. J. Jerry. Mapping genetic modifiers of mammary tumor susceptibility in BALB/c-p53+/- mice.

Frontiers in Cancer Prevention Research meeting of the AACR, Boston, October 2002.
. A. C. Blackburn, J. S. Brown, S. P. Naber, C. N. Otis and D. J. Jerry. *Cdkn2a* and *Prkdc* interact to modify tumor susceptibility in mammary tumor-prone Trp53+/- mice

American Society of Human Genetics. 2002.
Mechanisms of tumorigenesis in mammary tumors from BALB/c-Trp53-heterozygous mice: A model for Li-Fraumeni syndrome. R. Naeem, A.C. Blackburn, M.S. Mohammed, S.P. Naber, and D.J. Jerry.

Development of cell lines and tissue repositories:

ABV14 mouse mammary adenocarcinoma cell line has been developed and is being used by collaborators to investigate cell signalling pathways involved in tumor cell death.

A panel of DNA samples has been collected from the mapping mice population of mice and are available for investigation of further candidate genes which may be involved in susceptibility to mammary adenocarcinoma or other tumor types which occur in p53+/- mice..

Grants:

NHMRC (Australia) Howard Florey Centenary Research Fellowship.
``Deficiency of tyrosine catabolism genes and accumulation of associated metabolites in mouse mammary epithelium --- potential low penetrance modifiers of breast cancer susceptibility." July 2002 -- June 2004. $AUD136,628

The training received by the PI, Anneke Blackburn, during the course of this grant gave her the necessary skills to be successful in her application for a research fellowship in an independent area of breast cancer research.

Conclusions

The research is progressing well, however a new strategy for answering the question of when the wild-type allele of p53 is lost is needed. Recently, hyperplastic outgrowth lines from p53+/- mammary epithelium have been developed, from which tumors develop at a frequency of around 40% (3). Southern blotting analysis of the hyperplastic cells and tumor tissue from these outgrowths may be possible as an alternative strategy to investigate when during tumor progression the wild-type allele is lost.

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Appendix 1

Reprint of published review article is attached.

Available online http://breast-cancer-research.com/currentissue/

Review

Knockout and transgenic mice of *Trp53*: what have we learned about p53 in breast cancer?

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Abstract

The human p53 tumor suppressor gene *TP53* is mutated at a high frequency in sporadic breast cancer, and Li–Fraumeni syndrome patients who carry germline mutations in one *TP53* allele have a high incidence of breast cancer. In the 10 years since the first knockout of the mouse p53 tumor suppressor gene (designated *Trp53*) was published, much has been learned about the contribution of p53 to biology and tumor suppression in the breast through the use of p53 transgenic and knockout mice. The original mice deficient in p53 showed no mammary gland phenotype. However, studies using BALB/c-*Trp53*-deficient mice have demonstrated a delayed involution phenotype and a mammary tumor phenotype. Together with other studies of mutant p53 transgenes and p53 bitransgenics, a greater understanding has been gained of the role of p53 in involution, of the regulation of p53 activity by hormones, of the effect of mouse strain and modifier genes on tumor phenotype, and of the cooperation between p53 and other oncogenic pathways, chemical carcinogens and hormonal stimulation in mammary tumorigenesis. Both p53 transgenic and knockout mice are important *in vivo* tools for understanding breast cancer, and are yet to be exploited for developing therapeutic strategies in breast cancer.

Keywords: breast cancer, knockout, p53, transgenic

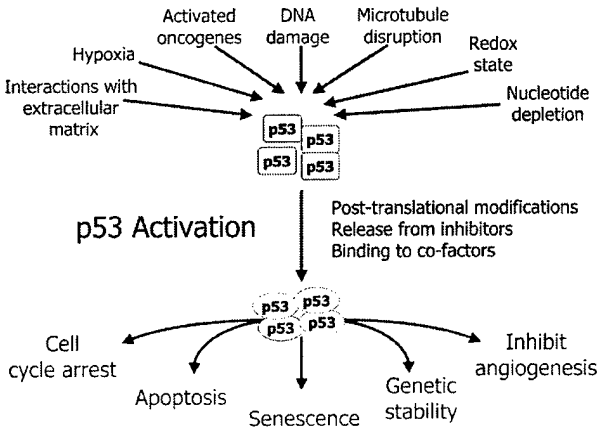
Introduction

The p53 tumor suppressor gene (*TP53* in humans or *Trp53* in mice) is critical for inhibiting tumor development in many tissues. This is evident in breast epithelium from the high frequency of mutations in *TP53* in sporadic human breast cancers [1]. Consistent with this phenomenon, germline mutations in p53 predispose women to breast cancer. Li–Fraumeni syndrome (LFS) patients, of whom approximately one-half carry mutations in one allele of *TP53* [2], suffer from a high frequency of breast cancer, with early-onset breast cancer accounting for approximately one-half of the tumors observed in LFS women [3,4]. Furthermore, even in the context of mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2*, high rates of p53 mutation are found [5,6]. Thus, p53 appears to be a critical agent in the protection of the breast epithelium against tumorigenesis.

The way in which p53 performs its tumor suppressor role involves diverse cellular processes. The repertoire of p53 activities includes the regulation of the cell cycle, apoptosis, senescence, facilitating DNA repair, and antagonizing angiogenesis (Fig. 1). Many of these functions are mediated by transcriptional activation of target genes by p53 [7],

DMBA = 7,12-dimethylbenz[a]anthracene; IL = interleukin; K14 = keratin 14; LFS = Li–Fraumeni syndrome; MMTV = mouse mammary tumor virus; *TP53* = human p53 tumor suppressor gene; *Trp53* = mouse p53 tumor suppressor gene; WAP = whey acidic protein; WAP-*DES* = WAP-*des(1–3)insulin-like growth factor-I* transgene.

Figure 1



Activation of p53 and responses. The p53 protein can exist in multiple states within cells. Under normal conditions, levels of p53 protein are very low due to rapid turnover. Activation of cellular stress pathways causes covalent modification and tetramerization of p53, stabilizing the protein and leading to accumulation within the nucleus. The cellular outcome depends on the balance of factors present in cells that may antagonize or promote particular responses.

such as the induction of cell-cycle arrest by transcriptional activation of $p21^{WAF1}$, a cyclin-dependent kinase inhibitor. Other activities may involve the p53 protein more directly, such as the $3' \rightarrow 5'$ exonuclease activity of p53 that may contribute to fidelity of DNA replication [8].

The p53 protein levels are normally very low, due to rapid turnover, but in response to various stressful stimuli, such as DNA damage or hypoxia, the p53 protein is stabilized and activated to perform its functions. The regulation of p53 stability and activity involves many proteins, such as the antagonist MDM2 that binds the transactivation domain and also targets p53 for ubiquitination and degradation, or the kinases DNA-PK and ATM that phosphorylate p53 at several of its many sites for post-translational modification [9,10]. The overall p53 response of a cell thus depends on the integration of the signals and activities of many different mechanisms and proteins. The important consequences of p53 activity, potentially cell death or tumor promotion, require this fineness and multiplicity of controls.

With this level of control over p53 function, it is not surprising that loss or mutation of one allele of p53 can have serious consequences. This is the case in LFS patients, who are susceptible to a wide range of tumor types in addition to breast cancer [3,4]. In contrast with Knudson's two-hit hypothesis for tumor suppressor genes, however, tumors can develop in $Trp53^{+/-}$ mice while still retaining a functional wild-type *Trp53* allele, indicating that p53 is haploinsufficient for tumor suppression [11]. The reduction in p53 dosage that results from heterozygosity at the genetic level has been shown in many mouse tissues to result in an intermediate level of transcriptional activity, growth control and apoptosis compared with wild-type and $Trp53^{-/-}$ cells [12–14]. This may lead to the retention of cells that have acquired further oncogenic mutations, thus resulting in haploinsufficiency for tumor suppres sion.

The protection of the mammary epithelium against tumorigenesis by p53 may thus involve many different processes. While the fine details of mechanisms and regulation are best addressed in highly manipulable *in vitro* systems, the biological consequences of such findings need to be verified *in vivo* where all the signals from the whole tissue and whole animal are intact. Studies in transgenic and knockout mice on the role of p53 and other molecules in breast cancer have therefore not focused on detailed mechanisms, but have been aimed at mimicking genetic susceptibility to breast cancer observed in humans, and at demonstrating a functional role for molecular lesions observed in end-stage human disease. While *Trp53* knockout mice have provided much insight into the role of p53 in tumor suppression, tools that allow specific modulation of the mammary gland have greatly enhanced, and in some cases have been essential to, our understanding of the function of p53 and its interaction with other molecules in protection against breast cancer.

Altering p53 function in the mouse mammary gland

The function of p53 has been modified genetically in mice in several ways (Table 1). Targeted disruption of the *Trp53* gene has been performed by several groups [15–17]. This process removes several exons from the endogenous gene and results in the absence of p53 protein in all tissues of the mouse throughout development and adulthood. Mice homozygous for the *Trp53* null allele ($Trp53^{-/-}$) die at 4–6 months of age, primarily from lymphomas. Only on the BALB/c genetic background have significant numbers of mammary tumors been observed in $Trp53^{+/-}$ mice, mimicking LFS of humans [18].

To study gene function in a particular tissue without the complication of death due to other tumor types (e.g. lymphomas) or embryonic lethality, tissue-specific deletion of genes within the mammary glands has been achieved using conditional knockouts [19,20]. In this system, the gene to be deleted is modified by inserting recombination sites (*loxP* or *FRT*) both sides of the gene, and it is introduced into the mouse as a targeted insertion. This does not alter gene function until a recombinase is expressed that recombines the chromosome at the two inserted recombination sites, thus deleting the gene segment between them. Tissue-specific expression of the recombinase (Cre for *loxP* sites, and Flp for *FRT* sites) achieves the tissue-specific gene deletion.

Table 1

Genetic alterations of the *Trp53* gene in mice

Modification	Promoter	Pregnancy required	Effect	References
p53 deficient, *Trp53*$^{-}$	–	No	No p53 protein	[15–17]
Mutant p53 transgenes				
p53-R172H	WAP	Yes	Dominant negative/gain of function	[33,40]
p53-R172L	WAP	Yes	Super active p53	[49,50]

WAP, whey acidic protein.

The following promoters are used to target the mammary gland: the whey acidic protein (WAP) promoter, which is turned on exclusively during pregnancy and lactation [19]; the mouse mammary tumor virus (MMTV) promoter, which is expressed constitutively in the mammary gland at low levels but also in a limited number of other tissues, including the salivary gland, and which is responsive to hormones [19]; and the human keratin 14 (K14) gene promoter, which is expressed in the epithelium of numerous tissues including the mammary gland and skin [21].

While K14 is expressed in myoepithelial cells and rarely in luminal epithelial cells of the mammary gland, expression of Cre under the K14 promotor resulted in lacZ reporter gene expression in both luminal and myoepithelial cells, possibly due to K14 promotor activity in a common progenitor [21]. It must be noted that promotors used to express recombinases may result in permanent genetic changes to cells that only transiently express the recombinase. Promotors must thus be carefully chosen considering their activity throughout development, not just in the adult tissue types.

While a molecular approa ch is one way to target modifications to the mammary gland, transplantation of mammary epithelium into cleared fat pads is a valuable alternative approach to analyze the effects of gene deletion in the mammary epithelium [22]. With this technique, the mammary ductal rudiments of 3-week-old (prepubescent) mice are surgically removed from the abdominal mammary glands. This leaves the fat pad 'cleared' of mammary epithelium into which donor epithelium can be placed. As the mouse matures, the donor epithelium will grow to fill the mammary gland.

Transplantation of *Trp53*$^{-/-}$ epithelium in this manner has demonstrated the role of p53 in protection against mammary tumor formation in the absence of other transgenes [23]. Furthermore, the absence of hormonal requirements to affect gene deletion or to promote transgene expression means that interactions between hormonal factors and the gene deletion can be studied. Contributions of hormonal simulation to mammary tumorigenesis in the absence of functional p53 have been studied in this manner [23,24].

While tissue-specific gene deletion has not been widely applied to studies of p53 function, overexpression of exogenous genes (i.e. transgenes) under tissue-specific promoters has been used to study p53 function in the mammary gland. p53 is a tetrameric protein, thus protein from a mutant allele can interact with and disrupt the function of p53 protein from a wild-type allele in a dominant-negative manner. This has been elegantly demonstrated in mice by the breeding of mice transgenic for mutant p53 (Val135) to both wild-type and *Trp53*$^{-/-}$ mice. The mutant transgene was able to accelerate tumor development in wild-type and *Trp53*$^{+/-}$ mice but had no effect in *Trp53*$^{-/-}$ mice, indicating its tumor promotion occurred only through disruption of wild-type p53 function [25].

Other mutant *Trp53* genes have been overexpressed selectively in the mammary gland under the control of the WAP promoter. These transgenes contain point mutations commonly found in *TP53* in human tumors, such as the *p53-R172H* mutant, which have dominant-negative or gain-of-function properties, and thus in some ways may better represent the human situation than complete absence of gene product [26]. However, the use of the WAP promoter, which is turned on exclusively during pregnancy and lactation, requires that the mice go through at least one and often multiple rounds of pregnancy and lactation to maintain transgene expression. An alternative approach is the use of the MMTV promoter that is expressed constitutively in the mammary gland, but this promoter is also responsive to hormones. Thus, when transgenes are driven by WAP or MMTV promoters, it is not possible to dissect hormonal events that may interact with p53 function in the mammary gland and affect tumorigenesis. To date, the MMTV promoter has not been used to introduce modified p53 genes. However, the MMTV promoter has been used to drive many mammary oncogenes that have been studied in combination with the *Trp53*$^{-}$ allele, as will be discussed (see Table 3 later).

Activity of p53 in normal mouse mammary epithelium

The occurrence of p53 mutations in breast cancer and the high rate of breast cancer in LFS patients pointed to an important role for p53 in the breast. Initial studies of p53-deficient mice (*Trp53*$^{+/-}$ and *Trp53*$^{-/-}$) [15–17] did not, however, reveal any essential function for p53 in the mammary gland. The p53-deficient mice did not develop mammary tumors, and both lactation and involution appeared normal. A closer examination at the cellular level of wild-type mice compared with *Trp53*$^{-/-}$ mice, however, revealed a role for p53-dependent apoptosis in the early stages of involution. Removal of pups from the wild-type lactating gland induced p21^{WAF1} expression, a p53 target gene, within 24–48 hours. This expression was absent in *Trp53*$^{-/-}$ mice. Correspondingly, involution in the *Trp53*$^{-/-}$ mice was delayed when examined in the early (days 2–5) weaning period [27]. Other workers have also reported observing fewer apoptotic cells at day 1 of involution in *Trp53*$^{-/-}$ mice compared with control mice [28]. Thus, p53 is important in inducing mammary epithelial apoptosis in response to a physiological stimulus.

The p53 protein is also important in the response of normal mammary epithelium to DNA damaging agents such as γ-radiation, but responsiveness is greatly affected by the hormonal status of the animal. In normal resting epithelium, whole-body irradiation of mice resulted in minimal increases in p53-dependent transcription of p21^{WAF1} and apoptosis. Treatment of the mouse with exogenous hormones (pregnant mare serum gonadotrophin and human chorionic gonadotrophin) prior to irradiation, however, produced a strong p53 response [29]. In the physiological setting, this regulation of p53 response by hormones has been demonstrated by the strong induction of p21^{WAF1} and apoptosis after irradiation during pregnancy and weaning, but by lack of response during lactation and in the virgin state [30]. The absence of p21^{WAF1} expression and apoptosis in similarly treated *Trp53*$^{-/-}$ animals demonstrated that these processes are p53 dependent.

This variation in p53 activity in the mammary gland involves many levels of regulation. The p53 mRNA levels are altered across developmental phases, suggesting hormonal regulation of transcription [27,29]. However, the virgin gland contains high levels of p53 mRNA and yet does not produce a p53 response to γ-radiation, indicating that translational and post-translational regulation are also important. Expression of antagonists of p53, such as MDM2, also varies across development stages (Pinkas and Jerry, unpublished data, 2002). However, this does not fully explain the variations in p53 activity. The mechanisms through which hormones modulate p53 function are yet to be determined.

The role of p53 in mammary tumor development in mouse models

Mammary tumor development in p53-deficient mice

While the original *Trp53*$^{-/-}$ mice developed normally, they succumbed to a range of tumors, primarily lymphomas, within 6 months. Mammary tumors were notably absent [15,16]. To examine the possibility that early death due to lymphoma may be obscuring mammary tumor development in *Trp53*$^{-/-}$ mice, BALB/c-*Trp53*$^{-/-}$ mammary epithelium was transplanted into cleared fat pads of wild-type hosts. In this model, spontaneous mammary tumors formed in 60% of outgrowths with a latency of 50 weeks [18,23], demonstrating that p53 deficiency can promote mammary tumor formation in mice as well as in humans (Table 2). The tumors formed were moderately to poorly differentiated adenocarcinomas with high levels of aneuploidy. The long latency and incomplete penetrance of the phenotype, however, indicated that other genetic events must be required in addition to the loss of p53 for tumors to form.

Trp53$^{+/-}$ mice survived much longer than *Trp53*$^{-/-}$ mice. They also succumbed to lymphomas and sarcomas, however, with mammary tumors accounting for less than 1% of tumors in 129/Sv and C57BL/6 x 129/Sv mouse strains [31]. In contrast, when the *Trp53*$^{-}$ allele was backcrossed onto the BALB/c strain (nine generations), which is known to be more susceptible to mammary tumorigenesis, spontaneous mammary tumors occurred in 55% of *Trp53*$^{+/-}$ female mice [18]. Genetic components cooperating with p53 deficiency must thus be present in BALB/c to promote mammary tumor formation, and BALB/c-*Trp53*$^{+/-}$ mice may serve as a unique model of LFS [18]. Similar to the *Trp53*$^{-/-}$ transplant tumors, these tumors were primarily adenocarcinomas, had a long latency of 8–14 months, and the majority were aneuploid. Furthermore, loss of the wild-type allele of *Trp53* occurred in all mammary tumors examined, suggesting that this is an important collaborating genetic event in tumor formation [18]. This is similar to observations in LFS, where loss of heterozygosity for *TP53* occurred at a high rate in breast cancers [32].

Studies using variations of these two models have explored the interactions between p53 and hormones, the mammary carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), and γ-radiation in mammary tumorigenesis (Table 2). The *Trp53*$^{-/-}$ transplant model has been used to examine the interactions between hormonal stimulation and p53 deficiency in mammary tumorigenesis. Chronic hormonal stimulation in the form of pituitary isografts increased tumor incidence from 60 to 100%, in addition to decreasing the latency (50 to 37 weeks) [23]. This observation indicates that, in the absence of p53, hormonal stimulation is a very potent tumorigenic stimulus. Indeed, further investigation has shown that low doses of

Table 2

p53 deficiency and mutation mammary tumor phenotypes

Model	Agent	Incidence (%)	Latency	Loss of heterozygosity	References
Trp53$^{-/-}$	Death from nonmammary tumors				[15,16,18]
BALB/c mammary epithelium transplants					
Trp53$^{+/+}$	NT or Pit Stim	0	–		[23]
Trp53$^{-/-}$	NT	61	50 weeks		[18,23]
Trp53$^{-/-}$	Pit Stim	100	37 weeks		[23]
Trp53$^{+/+}$	DMBA	4	26 weeks		[23]
Trp53$^{-/-}$	DMBA	60	35 weeks		[23]
Trp53$^{+/+}$	Pit Stim + DMBA	14	27 weeks		[23]
Trp53$^{-/-}$	Pit Stim + DMBA	90	25 weeks		[23]
Transgenic FVB WAP-*p53-R172H* mice					
p53-R172H	Pregnancy	10–15	> 1 year		[33]
Wild type	Pit Stim + DMBA	85	33 weeks		[33]
p53-R172H	Pit Stim + DMBA	100	24 weeks		[33]
Trp53$^{+/-}$					
B6x129 *Trp53*$^{+/+}$	Pit Stim + DMBA	35	5.0 months		[34]
B6x129 *Trp53*$^{+/-}$	Pit Stim + DMBA	40	4.6 months	No	[34]
BALB/c (N9) *Trp53*$^{+/-}$	Spontaneous	55	8–14 months	Yes	[18]
BALB/c or DBA/2 *Trp53*$^{+/+}$	γ-Radiation	0		–	[35]
DBA/2 (N1-6) *Trp53*$^{+/-}$	γ-Radiation	6		Yes	[35]
BALB/c (N1-6) *Trp53*$^{+/-}$	γ-Radiation	41		Yes	[35]

DMBA, 7,12-dimethylbenz[a]anthracene; NT, no treatment; Pit Stim, hormonal stimulation provided by pituitary isografts; WAP, whey acidic protein.

progesterone, but not estrogen, can promote aneuploidy in morphologically normal *Trp53*$^{-/-}$ mammary epithelial transplants [24].

Cooperation of the chemical carcinogen DMBA with p53 mutation or p53 deficiency has also been demonstrated in several situations. DMBA treatment alone decreased tumor latency (50 to 35 weeks) in *Trp53*$^{-/-}$ outgrowths [23]. When administered in addition to hormonal stimulation, latency was further reduced (25 weeks). Cooperation was also observed in mice carrying the *p53-R172H* transgene. This transgene, with continuous breeding to promote expression from the WAP promoter, resulted in only 10–15% spontaneous mammary tumor incidence in mice older than 1 year. However, using pituitary isografts to stimulate maximal transgene expression, the presence of the mutant *p53-R172H* transgene increased the tumor burden and incidence, and decreased the latency of tumors induced by DMBA administration [33]. A trend towards cooperation between *Trp53*$^{+/-}$ and DMBA treatment with pituitary stimulation has also been reported by Jerry *et al.* [34], but it was not statistically significant (Table 2).

Similar to spontaneous mammary tumor formation, the ability of γ-radiation to promote mammary tumorigenesis in *Trp53*$^{+/-}$ mice is greatly affected by the mouse strain background. With the *Trp53*$^{-}$ allele backcrossed one to six generations onto either the DBA/2 or BALB/c strain, γ-radiation significantly increased the incidence of mammary tumors in the BALB/c-*Trp53*$^{+/-}$ mice but not in the DBA/2-*Trp53*$^{+/-}$ mice [35]. This reinforces the importance of modifier genes that can alter phenotypes of tumor susceptibility mutations such as *Trp53*$^{-}$.

Loss of p53 function is critical in *Brca1*-associated and *Brca2*-associated breast cancer

In addition to LFS, the identification of the two breast cancer susceptibility genes, *BRCA1* and *BRCA2*, has led to many efforts to mimic in mice the susceptibility to breast cancer in humans incurred by mutations in these genes, and to understand the mechanisms by which they contribute to mammary carcinogenesis. Towards this, five strains of *Brca1* knockout mice have been generated (reviewed in [36]), revealing that complete *Brca1* deficiency results in embryonic lethality. *Brca1*$^{+/-}$ mice developed normally but, unlike humans carrying *BRCA1* mutations, failed to show increased susceptibility to mammary carcinomas, reminiscent of p53 heterozygous mice and LFS.

Using mice with germline mutations, Cressman *et al.* demonstrated cooperativity between p53 and *Brca1* in mammary tumor formation (Table 3), with 10% of

Table 3

Cooperation of p53 with other transgenes in mammary tumorigenesis

		Mammary tumor				
Transgene	*Trp53* genotype	Occurrence	Latency	Tumor *Trp53* status	Features	References
Brca1$^{+/-}$	+/+	No				[37]
	−/−	10%				[37]
Brca1$^{Ko/Co}$	+/+	Yes	10–13 months	Mutations		[38]
	+/-	Accelerated	6–8 months	80% LOH		[38]
Brca2$^{Co/Co}$	+/+	No				[21]
	+/−	Yes	360 days	100% LOH		[21]
	−/−	Accelerated	181 days			[21]
MMTV-*neu*	Wild type	Yes	234 days	Mutations	Euploid	[40]
	R172H	Accelerated	154 days		Aneuploid	[40]
WAP-*DES*	Wild type	Yes	21 months		Euploid	[41]
	R172H	Accelerated	14 months		Aneuploid	[41]
MMTV-*Wnt1*	+/+	Yes	22.5 weeks		Euploid	[42]
	+/−	Yes (no acceleration)	23.0 weeks	50% LOH		[42]
	−/−	Accelerated	11.5 weeks		Aneuploid, ↑ proliferation	[42]
					Less differentiated	[47]
MMTV-*ras*	+/+	Yes, and salivary tumors	8.5 months	No mutations		[43]
	+/−	Accelerated	6.3 months	No LOH, no mutations		[43]
	−/−	Few			↑ Aneuploidy, ↑ proliferation	[43]
		Salivary tumors dominate				[43]
MMTV/c-*myc*	+/+	Yes			Aneuploid	[51]
	+/−	Few		25% LOH	Aneuploid	[51]
	+/−	Lymphomas dominate		90% LOH		[51]

LOH, loss of heterozygosity; MMTV, mouse mammary tumor virus; WAP, whey acidic protein; WAP-*DES* = WAP-*des(1–3)insulin-like growth factor-I* transgene.

Brca1$^{+/-}$*Trp53*$^{-/-}$ mice developing mammary carcinomas compared with 0% of *Brca1*$^{+/-}$*Trp53*$^{+/+}$ mice [37]. With the development of conditional knockouts, Xu *et al.* were able to demonstrate more elegantly and sensitively the cooperation between these two genes. In mice with one germline and one conditional mutant allele of *Brca1* (*Brca1*$^{Ko/Co}$*MMTV-Cre*), mammary tumors developed after approximately 1 year. The addition of one germline mutation in *Trp53* (*Brca1*$^{Ko/Co}$*MMTV-CreTrp53*$^{+/-}$) accelerated mammary tumor formation to 6–8 months, and most of these tumors showed loss of the wild-type allele of *Trp53* [38]. Mutations in *Brca1* and *Trp53* are thus strongly cooperative in mammary tumorigenesis.

The first attempts to study *Brca2* knockout mice similarly found that the mutation was embryonic lethal. The recent generation of conditional *Brca2* knockout mice using K14 promoter-driven Cre expression, however, has allowed the study of *Brca2* deficiency in the mammary gland [21]. Strikingly, *K14cre;Brca2*$^{Co/Co}$ females developed no epithelial tumors over 900 days of monitoring, but the absence of p53 (also conditionally knocked out) resulted in all mice developing either skin or mammary carcinomas by 300 days of age. Deficiency of one *Trp53* allele was sufficient to promote mammary and skin tumor formation, and all tumors had lost the wild-type allele of *Trp53*. Disruption of the p53 pathway is thus pivotal in *Brca2*-associated breast cancer in mice [21]. This is consistent with human breast cancer, where mutations in p53 are seen in a large proportion of *BRCA1* and *BRCA2* breast cancers [5,6]. Interestingly, the majority of the mammary tumors observed in the *K14cre;Brca2*$^{Co/Co}$*Trp53*$^{Co/Co}$ model were carcinomas with basal or myoepithelial cell types, perhaps reflecting the dominant pattern of expression of the K14 promotor in myoepithelial cells [21].

p53 cooperates with many pathways involved in mammary tumorigenesis

In addition to breast cancer susceptibility genes, p53 has been studied for cooperativity with pathways activated in sporadic breast cancer. Transgenic mice overexpressing oncogenes involved in human breast cancer, such as HER-2/*Neu*, *Wnt1* and c-*myc*, have been generated that develop a high incidence of sporadic mammary carcinomas. By crossing these mice with *Trp53* mutant mice or null mice to generate double-transgenic mice, it has been possible to directly demonstrate pathway cooperativity (Table 3). For example, HER-2, a member of the epidermal growth factor receptor family, is overexpressed in approximately 20% of human breast cancers and is associated with a poor prognosis [39].

Transgenic mice expressing wild-type rat *neu* under the MMTV promoter (MMTV-*neu*) develop mammary tumors with an average latency of 234 days that have a high frequency of missense mutations in *Trp53* [40]. The cooperativity between the Neu and p53 pathways was clearly demonstrated by introducing the mutant p53 transgene, *p53-R172H*, into these mice, which reduced tumor latency to 154 days [40]. This study in transgenic mice has thus demonstrated strong cooperativity between two genetic lesions common in human breast cancer, and has demonstrated that p53 mutation is an important event in Neu-mediated oncogenesis.

Similar cooperativity was observed between p53 and the insulin-like growth factor pathway. Mice overexpressing an insulin-like growth factor-I analog, the WAP-*des(1–3)IGF-I* transgene (WAP-*DES*), demonstrated an 80% mammary tumor incidence with a latency of 21 months. This is accelerated to only 14 months when combined with the mutant p53 transgene *p53-R172H* [41]. Cooperativity between p53 and the Wnt pathway was examined with the $Trp53^{-}$ allele. Female mice transgenic for the *Wnt1* mammary oncogene develop mammary tumors with a median latency of 23 weeks. Inheritance of a single defective allele of *Trp53* did not confer any increased susceptibility in this model, but the latency was halved to 11.5 weeks with two germline $Trp53^{-}$ alleles [42]. In MMTV-*ras* mice, which develop both mammary tumors and salivary tumors, being $Trp53^{+/-}$ decreased tumor latency from 8.5 to 6.3 months with little change in the tumor spectrum [43].

In addition to decreasing tumor latency, defects in the p53 pathway in the aforementioned models altered the histological grade of the mammary tumors. While the *neu* tumors were euploid, *neu/p53-R172H* bitransgenic tumors were of a higher histological grade and exhibited aneuploidy [40]. WAP-*DES/p53-R172H* bitransgenic tumors showed a higher proportion of tumors with aneuploidy, and in those tumors they showed a higher proportion of aneuploid cells than in WAP-*DES* transgenic tumors [41]. Similar changes were observed in both salivary and mammary tumors of the MMTV-*ras* with loss of p53 [43]. In the *Wnt1* model, mammary tumors lacking p53 showed increased genomic instability with aneuploidy, amplifications, and deletions as shown by karyotype analysis and comparative genomic hybridization [42]. A common feature of mammary tumors lacking functional p53, regardless of the driving oncogenic force, is therefore genomic instability, which is consistent with the profile of p53 as 'guardian of the genome'.

The p53 genotype also affects the tumor growth rate in several of these models. Mammary tumors from *Wnt1*/$Trp53^{-/-}$ mice show more rapid growth once established compared with *Wnt1* tumors with functional p53. This was not due to decreased apoptosis, as there was no effect of p53 genotype on apoptosis levels. However, $Trp53^{-/-}$ tumors contained more mitotic figures and had more cells in the S phase than did *Wnt1* tumors suggesting elevated proliferation rates as an underlying defect [44]. Higher rates of proliferation were also observed in MMTV-*ras*/$Trp53^{-/-}$ bitransgenic tumors compared to MMTV-*ras* alone with no alteration in apoptosis levels [43]. This contrasts with animal model studies in other tissues, where accelerated tumor growth due to p53 loss occurred primarily through loss of normal apoptotic function [45,46]. The mechanisms by which p53 loss influences tumor progression may thus differ depending on tissue type and/or the oncogenic pathways involved.

The mechanism by which p53 inhibits tumor growth in the *Wnt1* model has been investigated. Expression profiling of *Wnt1* and *Wnt1*/$Trp53^{-/-}$ mammary tumors demonstrated that $Trp53^{-/-}$ tumors showed altered expression of several proliferation regulatory genes. Expression of $p21^{WAF1}$ [47], a p53 target gene, was decreased in tumors from *Wnt1*/$Trp53^{-/-}$ mice compared to *Wnt1* mice. Consistent with this pathway, *Wnt1*/$p21^{WAF1+/-}$ mammary tumors displayed increased tumor growth rates compared with *Wnt1* tumors [48]. The inhibition of tumor growth by p53 may thus be mediated by $p21^{WAF1}$.

While some effects of p53 on tumor phenotype have been clearly identified, the mechanisms by which p53 protects against cancer in normal mammary glands are more difficult to elucidate. A 'superactive' mutant p53 transgene, *p53-R172L*, expressed under the WAP promoter results in elevated apoptosis rates in normal mammary glands [49,50]. Interestingly, this transgene delayed mammary tumor onset induced by DMBA [50] and in transgenic mice expressing transforming growth factor α [26]. In these models, protection against breast cancer by p53 may thus be due to removal of genetically damaged cells by p53-dependent apoptosis.

In contrast, hyperplasias in *Wnt1* mammary glands showed no alteration in apoptosis rates with *Trp53* genotype [44], suggesting that p53-mediated apoptosis is not involved in tumor prevention in this model. Furthermore, in this same model, deficiency in $p21^{WAF1}$ (*Wnt1/p21*$^{WAF1-/-}$ and *Wnt1/p21*$^{WAF1+/-}$ mice) was unable to mimic the acceleration due to loss of p53 [48]. In the *Wnt1* model, therefore, it appears that protection against tumorigenesis by p53 occurs through mechanisms other than apoptosis or $p21^{WAF1}$-mediated cell-cycle arrest, such as maintaining genomic stability.

While p53 cooperates with many models, this is not the case in all models. Female mice carrying the MMTV/c-*myc* transgene develop mammary carcinomas at 8–10 months of age, with occasional lymphomas developing in males. When only one p53 allele is present, however, mice develop aggressive lymphomas that appear to require loss of the wild-type allele of p53. In contrast, there was no sign of acceleration of mammary tumors, and three-quarters of the mammary tumors that did form had retained the wild-type allele [51], indicating that cooperativity between c-*myc* and p53 is dependent on tissue type. Regardless of p53 genotype, c-*myc* tumors showed changes in ploidy and chromosomal aberrations [52]. The lack of tumor acceleration in the presence of c-*myc*-induced chromosomal instability and the lack of a role for apoptosis and $p21^{WAF1}$ cell-cycle arrest in the *Wnt1* model suggest that maintenance of genomic stability may be a major role for p53 in the prevention of mammary tumors.

SV40 viral oncogene inactivation of p53

The function of p53 protein may also be abrogated by expression of the SV40 viral oncogenes. While these viral proteins are not involved in human breast cancer, the viral proteins mimic alterations etiologically linked with human breast cancer. For this reason, several transgenic mice have been generated for studying mammary tumorigenesis [53–57]. However, there are several complications with these models in terms of understanding p53 function in breast cancer.

The first SV40 transgenics contained the SV40 early region, which produces multiple gene products including the large T antigen that functionally inactivates both pRb and p53, and including the small t antigen that inhibits the serine/threonine phosphatase PP2A. These models therefore do more than just interfere with p53 function, and it is only recently that the contribution of the different components of these gene products has been fully investigated.

A recent study by Goetz *et al.* [58] generated several WAP-SV40 transgenic lines that produce either large T antigen only (WAP-SVT), small t antigen only (WAP-SVt), or N-terminal truncated large T antigens (WAP-SVBst-Bam). All of these lines developed mammary tumors with a lower incidence than the original mice expressing both large T and small t antigens (SVT/t) (100, 64, 6–12 and 0% for SVT/t, SVT, SVt and SVBst-Bam, respectively) [57,58], indicating many cooperating functions in the SV40 early genome. Of particular note, however, is the lack of mammary tumors due to the SVBst-Bam transgene that retains the recognized pRb-binding and p53-binding portions of the SV40 genome. In contrast, small t antigen alone was able to result in tumor formation. The p53 and pRb binding activities of large T antigen thus require cooperation with small t antigen and the N-terminal region of large T antigen to induce mammary tumor formation [58].

p53 and metastases, chemotherapy and gene therapy

Most mouse models of breast cancer do not display metastatic disease. However, mice expressing the SV40 early genome under the control of the C3(1) component of the rat prostatic steroid binding protein promoter (C3(1)/TAG) [56] develop very aggressive mammary tumors in only 16 weeks, with around 15% showing lung metastases.

In this model, mice being *Trp53*$^{+/-}$ did not significantly alter latency or lead to loss of the remaining allele of *Trp53*. It did, however, result in more aggressive mammary tumors, as evidenced by increased numbers and sizes of metastases, than did TAG-*Trp53*$^{+/+}$ mice [59]. Thus, loss of p53 results in higher grade tumors, more aneuploidy in the primary tumor and promotes metastases. This is consistent with the poorer prognosis of patients with p53-negative/mutated tumors versus those without p53 mutations. The protection against metastases by p53 may be mediated by its induction of anti-angiogenesis genes, but these characteristics of p53 have not been fully explored in the transgenic/knockout mouse models to date.

The widely held tenet of chemotherapy is that cells die due to apoptosis, and that the absence of p53 may result in resistance to apoptosis and hence to chemotherapy. However, this has not been demonstrated clearly for non-hematological tumors [60]. Indeed, the p53-mediated apoptotic response to γ-radiation of mouse tumors was shown to vary among tissues [61]. In DMBA-induced mouse mammary tumors, whole-body irradiation was able to induce nuclear p53 expression and apoptosis. This contrasted other tumor types, such as liver and lung adenomas, where neither p53 expression nor apoptosis was induced by radiation. Thus, while the role of p53 in inducing apoptosis in response to radiation (and probably other chemotherapeutic agents) varies among tissue and tumor types, it would appear that mammary tumors retaining wild-type p53 are one type in which p53 is important [61].

Barrington *et al.* [62] have made use of transgenic mouse models to examine the role of p53-mediated apoptosis in

tumor regression *in vivo* of several chemotherapeutic agents. L-744,832, a potent and selective inhibitor of farnesyltransferase that catalyses an obligatory step in Ras protein activation, leads to the rapid regression of mammary and salivary tumors in MMTV-*ras* transgenic mice. This occurred by inducing high levels of apoptosis, and tumors arising in *ras*/*Trp53*$^{-/-}$ mice regressed at least as efficiently as *ras*/*Trp53*$^{+/+}$ mouse tumors, indicating that the response was largely p53-independent [62]. This contrasts with the effectiveness on *ras*/*Trp53*$^{-/-}$ salivary tumors of doxorubicin, which was not able to inhibit tumor growth, and of paclitaxel, which inhibited growth but did not result in regression [63]. Yet both these agents were effective in *ras*/*Trp53*$^{+/+}$ tumors. Interestingly, neither doxorubicin nor paclitaxel efficiently induced apoptosis but, rather, they changed the distribution of cells in the cell cycle. The p53 knockout and transgenic models can thus be used to examine mechanisms and effectiveness of therapeutic agents in the presence and absence of p53. The use of mouse models in this way will probably provide valuable information for the clinic on the tailoring of therapeutic agents that target particular molecular lesions of cancers.

In addition to altering the cell cycle and apoptosis as a means of mediating treatment efficacy, the expression of p53 in mammary tumors can also alter the efficacy of immunomodulatory gene therapy. In mice bearing PyMT-induced tumors, intratumoral injection of adenoviruses carrying either IL-2 or p53 was able to delay tumor growth, but when treated with both viruses together, regression in 65% of tumors without IL-2 toxicity was obtained [64]. The mechanisms by which p53 enhances this treatment are unclear, but may be due to growth inhibition allowing expansion of the cytotoxic T-lymphocyte response towards the tumors. Only a small percentage of tumor cells in this system are infected by the adenovirus, so while the anti-tumor growth may be mediated by apoptosis, it is likely to also involve a large bystander effect, and perhaps anti-angiogenesis mechanisms [64].

Conclusions

While initial studies of *Trp53* knockout mice did not suggest that p53 was important in the mouse mammary gland, it is now clear that p53 has important roles in normal physiology and tumorigenesis in the mouse. Knockout and transgenic mice with modified p53 functions will thus be useful tools in understanding breast cancer biology and response to therapies. p53 can mediate apoptosis in the mammary gland in response to both physiological and exogenous stresses. Deficiency of p53 alone, in a susceptible genetic setting, is sufficient to allow mammary tumor development in mice, and it has also been demonstrated to be pivotal in breast cancer formation in the context of *Brca1* and *Brca2* deficiency. We are therefore now able to model in mice three strong breast cancer susceptibility syndromes of humans.

Deficiency of p53 modifies tumor biology, promoting aggressiveness, rapid growth and genetic instability. p53 deficiency in the mouse cooperates with other oncogenic events important to human breast cancer, accelerating mammary tumor formation. Identification of further pathways (and hence modifier genes) with which p53 deficiency cooperates, combined with understanding mechanisms that regulate p53 activity, will greatly enhance our understanding of breast cancer biology and our ability to prevent it. With the complexity of tumor biology and the paracrine activities of p53, such as anti-angiogenic activities, the use of p53 transgenic and knockout mice provides important *in vivo* tools for understanding and developing therapeutic strategies.

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